Immunomodulatory effect of recombinant human procalcitonin on mitogenic activity of lymphocytes

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Abstract

Procalcitonin (PCT) is an excellent inflammatory marker for early diagnosis of sepsis. However, little is known about its immunological functions.

Objective: *The aim of our work was to determine the effect of recombinant human (rh) PCT on blastic transformation of lymphocytes.*

Methods: *Peripheral blood lymphocytes from 9 healthy volunteers were isolated on Ficoll-Metrizoat gradient and cultivated with polyclonal mitogens phytohemaglutinin (PHA), concanavalin A (Con A) and lipopolysaccharide (LPS) with or without rhPCT. RhPCT was added to the cultures in four different final concentrations.*

Results: *1. RhPCT significantly inhibited the blastic transformation of PHA activated T-lymphocytes (by 14-26%) as well as the simultaneous activation of T cells with rhPCT and PHA (21%). 2. RhPCT significantly decreased the ability of T cells stimulated by Con A either simultaneously (13-19%) or subsequently (21-41%). 3. RhPCT increased the activity of unstimulated autologous lymphocytes.*

Conclusion: *Our preliminary results indicate that rhPCT suppressed the activity of both PHA and Con A stimulated T-cells, however it increased the activity of unstimulated lymphocytes. The results indicate that in vivo production of PCT in septic patients might contribute to a suppression of the hyperactivated specific cellular immunity.*

Key words: *immunity, lymphocytes, mitogens, procalcitonin, sepsis.*

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Introduction

Procalcitonin (PCT) is one of the calcitonin precursors synthesised in thyroidal C-cells and other neuroendocrine cells. It was originally described as a 116-amino acid protein with a relative molecular mass of 14.5 kDa in 1984 [1] and its gene (Calc-I) was located on chromosome 11p15.4 and sequenced in 1989 [2].

Under basal conditions, calcitonin precursors are produced mainly in neuroendocrine C-cells of the thyroid gland. In the absence of infection, the extra-thyroidal transcription of the Calc-I gene is suppressed and confined to selective expression in neuroendocrine cells found mainly in thyroid and lung [3, 4].

PCT and other calcitonin precursors in plasma/serum are present at concentrations less than 0.5 ng . ml^{-1} in healthy individuals most frequently under the detection limit of used diagnostic methods, but are highly elevated in sepsis [5-8].

A microbial infection induces an ubiquitous increase in Calc-I gene expression and a release of calcitonin precursors from different tissues and cell types throughout the body. High plasma levels of PCT are observed in patients with severe sepsis and the hepatosplanchnic region has been suggested to be a possible important tissue source [9, 10]. Meisner et al. show for the primary role for the liver as a source of PCT production in sepsis [11]. Various human tissues differ in the proportion of PCT production. Organs

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and tissues with the highest PCT concentration after LPS injection in a primate model included liver, kidney, aorta, fat tissue, ovaries, bladder and adrenal gland [12-14]. These data confirmed an extrathyroidal origin of PCT. Peripheral blood mononuclear cells may be also among the sources of elevated PCT levels in patients with sepsis [15, 16]. Thus under septic circumstances, the entire body could be viewed as an endocrine gland [4].

Clinically, the determination of the levels of circulating PCT and other calcitonin precursors is useful in complex, polymorbid cases with suspected bacterial infections. Multiple clinical studies have demonstrated the diagnostic utility of serum PCT levels for early diagnosis of bacterial sepsis [7, 8, 17] and its considerable utility for monitoring the severity of sepsis, organ failure and risk of mortality [18-20]. One can suggests that PCT in sepsis could play some role in defence mechanisms or to suppress the exaggerated immune response. The aim of our study was to evaluate the effect of rhPCT on mitogenic activity of human lymphocytes.

Material and methods

Peripheral blood was taken from nine healthy donors. Lymphocytes were isolated from heparinized venous blood by separation on a Ficoll-Metrizoat gradient and resuspended (10^6 cells/ml) in RPMI 1640 medium (Gibco), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamycin and 10% of heat inactivated pooled human serum. The cultures were set up in triplicates using "U"- bottom 96 micro-well plates (Nunc). To each well of the 96-well plate containing lymphocyte suspension (100 000 lymphocytes in 100 µl RPMI 1640 medium) either 50 µl of RPMI medium (non-stimulated cells) or 50 µl of phytohaemagglutinin (PHA; Sigma; 50 µl; dilution 1:10 in RPMI) or 50 µl of concanavalin A (Con A; Sigma; 2 µg/50ml) or lipopolysaccharide (LPS; Sigma; 1 µg) were added. Recombinant human procalcitonin (rhPCT; Brahms GmbH) was diluted with PBS supplemented with 2% BSA and added to the cultures (50 µl) in 4 different final concentrations: PCT1 – 9 ng . ml^{-1}, PCT2 – 55 ng . ml^{-1}, PCT3 – 295 ng . ml^{-1}, PCT 4 – 555 ng . ml^{-1}. The test was set up in three different modifications: 1., Lymphocytes were first pre-incubated with mitogens (PHA, CON-A or LPS respectively) and rhPCT was added to the cultures 4 hours later. 2., Both rhPCT and mitogen were simultaneously added to lymphocytes of the beginning of the test. 3., Lymphocytes were first pre-incubated with different concentrations of rhPCT (PCT1, PCT2, PCT3, PCT4) for 4 hours and polyclonal mitogens were added to them afterwards. Lymphocyte cultures were cultivated at 37°C in a CO_2 incubator (Juan, France) in 5% humified $C0_2$ atmosphere. 37 kBq of ^{3}H-thymidin ([^{3}H]TdR; Amersham, UK) in 20 µl of RPMI was added to each well on the day 3 and the cultivation proceeded for next 8 hrs when cultures were harvested to glass fibre filters Whatman (GF/C) by Auto-Mash (Dynex). The amount of incorporated [^{3}H]TdR into DNA of proliferating lymphocytes was determined by liquid scintillation counting on a beta counter (Spectral, LKB). The results were expressed in desintegrations per minute (dpm). Absolute values measured for individual donors were transformed to relative values and are expressed as percentage of inhibition/stimulation of cell proliferation as compared to control lymphocytes (without adition of rhPCT).

Statistical analysis

One sample test was used for a statistical evaluation. Statistical null hypothesis means that the mean value of a marker investigated (e.g. PCT1) is equal 100%. A value „t" is the value of a criterion tested (the Student´s test for one sample), „df" is the degree of freedom (n-1), two – tailed significance is a risk of rejection of the null hypothesis. The null hypothesis is usually rejected at a significance level <0.05. However, when the investigated cohort is too small, as it is in our case, even at big differences between „etalon" (in our case 100%) and a mean in the cohort, the test has not to show the low level of statistical significance. Therefore the effect size has been used. One of its markers is Cohen's „d". The greater the value is, the more relevant is the difference between the mean and the 100% value. Even the difference is not statistically significant, the effect – medical significance, as the value „d" indicates, is relevant. The values „d" diverge from 0 without upper limits: 0.20 means low, 0.50 medium, and 0.80 and more great levels of effect size significance.

Results

1a. Preincubation of lymphocytes with PHA for 4 hours. RhPCT (PCT1, PCT2, PCT3, PCT4) was added to the cultures afterwards (figure 1, tables 1, 2).

The aim of this part of experiment was to find out whether rhPCT influences the proliferation of PHA activated lymphocytes. We found that rhPCT had statistically significantly inhibited the function of PHA activated lymphocytes (PCT1: p=0.0001; d=2.75; PCT2: p=0.05; d=1.44; PCT3: p=0.04; d=1.49; PCT4: d=0.58).

1b. Co-cultivation of rhPCT and PHA with lymphocytes from the beginning of the test (figure 2, tables 1, 2).

This part of experiment aimed to find out whether rhPCT influences anyway the simultaneous activation of lymphocytes with PHA. Our results showed a decrease of lymphocytes activation in the presence of PHA. The strongest inhibition (statistically significant) was seen in the presence of rhPCT2 (p=0.004; d=1.48). Inhibitory activities of other concentrations of rhPCT were statistically not significant, however the Cohen's d of medical significance was high (PCT1: d=0.81; PCT3: d=0.80).

1c. Lymphocytes were first preincubated with rhPCT (PCT1, PCT2, PCT3, PCT4) for 4 hours. PHA was added to the cultures afterwards (figure 3, tables 1, 2).

Table 1. Results of experiments

Type of experiments	-PCT [%]	PCT1 [%]	PCT2 [%]	PCT3 [%]	PCT4 [%]
Ly + PHA (PCT after 4 hours)	100	85.5	74.3	76.9	88.7
Ly + ConA (PCT after 4 hours)	100	120.3	115.1	108.6	94.9
Ly + LPS (PCT after 4 hours)	100	98.2	108	130.4	143.02
Ly+ PHA + PCT (simultaneously)	100	86.58	78.67	90.95	97.78
Ly + ConA + PCT (simultaneously)	100	87.29	81.04	86.2	94.67
Ly + LPS + PCT (simultaneously)	100	115.41	132	79.48	116.08
Ly + PCT (PHA after 4 hours)	100	91.74	89.67	87.95	106.25
Ly + PCT (ConA after 4 hours)	100	71.26	70.32	59.26	78.51
Ly + PCT (LPS after 4 hours)	100	142.08	88	92.6	135.76
Ly +PCT (simultaneously)	100	126.9	148.8	213.8	129.3
Ly + PCT (PCT after 4 hours)	100	141.78	161.06	130.56	101.96

Ly – lymphocytes, PHA – phytohemagglutinin, ConA – concanavalin A, PCT – procalcitonin, LPS – lipopolysaccharide, -PCT – cultures without PCT, concentration of PCT1 – 9 ng . ml^{-1}, PCT2 – 55 ng . ml^{-1}, PCT3 – 295 ng . ml^{-1}, PCT4 – 555 ng . ml^{-1}.

The aim of this part of study was to find whether pretreatment of lymphocytes with rhPCT influences the subsequent activation of these cells in the presence of PHA.

Rh-PCT only slightly depressed the subsequent activation of lymphocytes with PHA (PCT1: d=0.23; PCT2: d=0.27; PCT3: d=0.42; PCT4: d=0.31). Changes were statistically not significant.

2a. Lymphocytes were first preincubated with ConA. rhPCT (PCT1, PCT2, PCT3, PCT4) was added to the cultures 4 hours later (figure 1, tables 1, 2).

RhPCT added 4 hours later to Con A preactivated lymphocytes slightly increased the activity of these cells. Results were not statistically significant. The Cohen's "d" showed only low or mean value of medical significance (PCT1: d=0.57, PCT2: d=0.44, PCT3: d=0.24).

2b. RhPCT and Con A simultaneously added to lymphocytes from the beginning of the test (figure 2, tables 1, 2).

RhPCT led to statistically significant inhibition of lymphocytes activation in the presence of Con A (PCT1: p=0.001, d=1.66; PCT2: p=0.001, d=1.72; PCT3: p=0.05, d=1.09).

2c. Lymphocytes were first preincubated with rhPCT (PCT1, PCT2, PCT3, PCT4) for 4 hours. Afterwards Con A was added to the cultures (figure 3, tables 1, 2).

The greatest influence of rhPCT was found in this part of experiments. Our results showed that preincubation of lymphocytes with rhPCT had prevented from subsequent activation of these cells by Con A (PCT1: p=0.001, d=1.75; PCT2: p=0.003, d=1.39; PCT3: p=0.000, d=3.28; PCT4: p=0.005, d=1.29).

3. The influence of rhPCT and LPS on lymphocytes (figures 1-3, tables 1, 2).

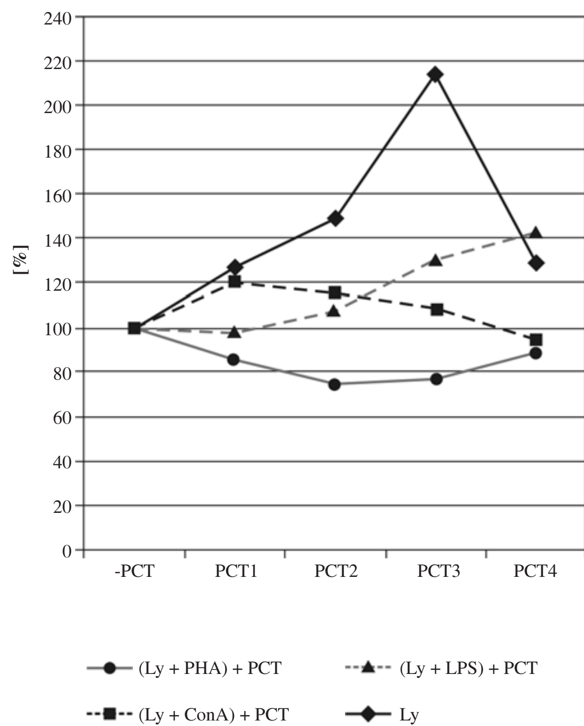

Fig. 1. Preincubation of lymphocytes with mitogens (PHA, ConA, LPS) for 4 hours. RhPCT was added to the cultures afterwards. Ly – lymphocytes, PCT – procalcitonin, PHA – phytohaemagglutinin, ConA – concanavalin A, LPS – lipopolysaccharide, -PCT – cultures without PCT, concentration of PCT1 – 9 ng . ml^{-1}, PCT2 – 55 ng . ml^{-1}, PCT3 – 295 ng . ml^{-1}, PCT4 – 555 ng . ml^{-1}.

Table 2. Results of statistical analyses

		One sample test			
		Test value = 100%			
		T	**df**	**Sig. (2 – tailed)**	**d**
1. Ly + PHA + PCT after 4 hours	PCT1 [%]	-7.783	8	**0.0001**	**2.75**
	PCT2 [%]	-4.085	8	**0.005**	**1.44**
	PCT3 [%]	-4.216	8	**0.004**	**1.49**
	PCT4 [%]	-1.639	8	0.145	0.58
2. Ly + PHA + PCT (simultaneously)	PCT1 [%]	-2.284	8	0.056	**0.81**
	PCT2 [%]	-4.178	8	**0.004**	**1.48**
	PCT3 [%]	-2.256	8	0.059	0.8
	PCT4 [%]	-0.46	8	0.659	0.16
3. Ly + PCT + PHA after 4 hours	PCT1 [%]	-0.703	8	0.502	0.23
	PCT2 [%]	-0.796	8	0.449	0.27
	PCT3 [%]	-1.266	8	0.241	0.42
	PCT4 [%]	-0.937	8	0.376	0.31
4. Ly + ConA + PCT after 4 hours	PCT1 [%]	1.722	8	0.123	0.57
	PCT2 [%]	1.334	8	0.219	0.44
	PCT3 [%]	0.722	8	0.491	0.24
	PCT4 [%]	-0.37	8	0.721	0.21
5. Ly + ConA + PCT (simultaneously)	PCT1 [%]	-4.99	8	**0.001**	**1.66**
	PCT2 [%]	-5.157	8	**0.001**	**1.72**
	PCT3 [%]	-3.259	8	**0.012**	**1.09**
	PCT4 [%]	-1.003	8	0.345	0.33
6. Ly + PCT + ConA after 4 hours	PCT1 [%]	-5.263	8	**0.001**	**1.75**
	PCT2 [%]	-4.176	8	**0.003**	**1.39**
	PCT3 [%]	-9.837	8	**0.0001**	**3.28**
	PCT4 [%]	-3.881	8	**0.005**	**1.29**
7. Ly + LPS + PCT after 4 hours	PCT1 [%]	-0.089	7	0.932	0.03
	PCT2 [%]	0.218	7	0.833	0.08
	PCT3 [%]	0.78	7	0.461	0.28
	PCT4 [%]	1.498	7	0.178	0.53
8. Ly + LPS + PCT (simultaneously)	PCT1 [%]	1.775	7	0.119	0.63
	PCT2 [%]	1.007	7	0.348	0.36
	PCT3 [%]	-3.939	7	**0.006**	**1.39**
	PCT4 [%]	1.919	7	0.096	0.68
9. Ly + PCT + LPS after 4 hours	PCT1 [%]	1.152	8	0.283	0.38
	PCT2 [%]	-1.035	8	0.331	0.34
	PCT3 [%]	-0.5	8	0.63	0.17
	PCT4 [%]	1.965	8	0.085	0.66
10. Ly + PCT (simultaneously)	PCT1 [%]	1.658	7	0.141	0.59
	PCT2 [%]	2.499	7	**0.041**	**0.88**
	PCT3 [%]	1.43	7	0.196	0.51
	PCT4 [%]	0.12	7	0.908	0.04
11. Ly + PCT after 4 hours	PCT1 [%]	0.868	8	0.411	0.29
	PCT2 [%]	1.631	8	0.142	0.54
	PCT3 [%]	2.706	8	**0.027**	**0.9**
	PCT4 [%]	1.272	8	0.239	0.42

PCT – procalcitonin, PHA – phytohaemagglutinin, ConA – concanavalin A, LPS – lipopolysaccharide, PCT1 – 9 ng . ml^{-1}, PCT2 – 55 ng . ml^{-1}, PCT3 – 295 ng . ml^{-1}, PCT4 – 555 ng . ml^{-1}.

These experiments were also set up in three modifications. The effect of LPS on lymphocytes proliferation was not uniform (figures 1-3). The greatest differences in the activity of lymphocytes were found in experiments with simultaneous cultivation of lymphocytes with both rhPCT and LPS (inhibition of lymphocyte proliferation: p=0.006, d=1.39).

4. The influence of rhPCT on autologous lymphocytes (figures 1-3, tables 1, 2).

Our results showed stimulatory activity of rhPCT on autologous (non-stimulated) lymphocytes (i.e. without mitogens). The cultivation of rhPCT with lymphocytes led to their increased activity in both types of the tests performed: co-cultivation of lymphocytes with rhPCT from the beginning of the test and when rhPCT was added to cultured lymphocytes 4 hours later.

Finally, our results showed that rhPCT increased the proliferation of unstimulated lymphocytes, but decreased the activity of PHA preactivated T cells as well as the simultaneous activation of T cells with both rhPCT and PHA. RhPCT decreased also the ability of T lymphocytes to be stimulated by Con A either simultaneously or subsequently.

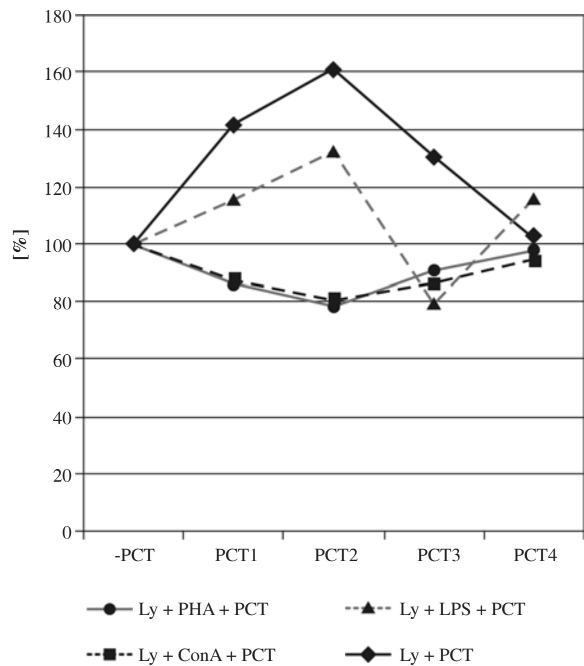

Fig. 2. Cocultivation of lymphocytes, mitogens and rhPCT from the beginning of the test. Ly - lymphocytes, PCT – procalcitonin, PHA – phytohaemagglutinin, Con A – concanavalin A, LPS – lipopolysaccharide, -PCT – cultures without PCT, concentration of PCT1 – 9 ng . ml^{-1}, PCT2 – 55 ng . ml^{-1}, PCT3 – 295 ng . ml^{-1}, PCT4 – 555 ng . ml^{-1}.

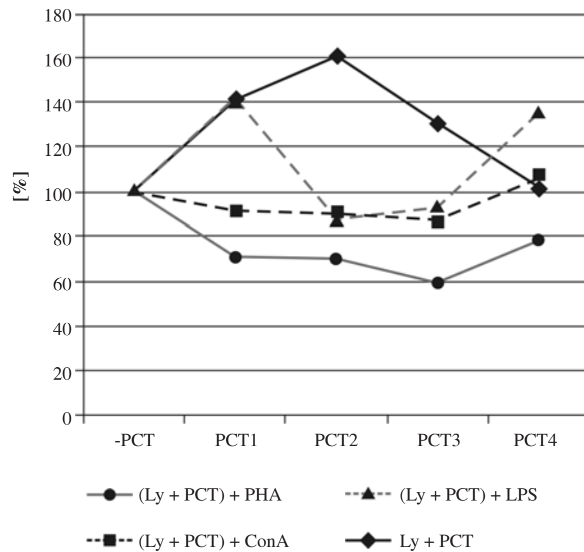

Fig. 3. Lymphocytes were first preincubated with rhPCT. Mitogens were added to the cultures 4 hours later. Ly – lymphocytes, PCT – procalcitonin, PHA – phytohaemagglutinin, ConA – concanavalin A, LPS – lipopolysaccharide, -PCT – cultures without PCT, concentration of PCT1 – 9 ng . ml^{-1}, PCT2 – 55 ng . ml^{-1}, PCT3 – 295 ng . ml^{-1}, PCT4 – 555 ng . ml^{-1}.

Discussion

Procalcitonin is an excellent inflammatory marker for the early diagnosis of sepsis with a good prognostic value. However, its physiological and immunological functions are not very well understood. PCT was found to be an ancient humoral component of inflammatory immune response to severe systemic bacterial infection and sepsis [21-23]. Its gene induction (PCT mRNA) and protein synthesis is directly coupled to the systemic effect of endotoxin (LPS) or other bacterial components and key pro-inflammatory cytokines [15, 16, 24]. In humans and experimental animals, serum levels of calcitonin precursors, including PCT increase several fold to several thousand-fold, and this increase often correlates with the clinical diagnosis of sepsis, its severity, and prognosis [25]. Blood concentrations of PCT are increased in systemic inflammation, especially when this is caused by bacterial, parasitic or fungal infections [8, 18, 26].

In addition of being a marker of sepsis, PCT plays a critical role as a mediator of systemic infections and contributes markedly to deleterious effects of sepsis. High concentrations of serum PCT found in sepsis persist for a relatively long period of time and may augment and amplify the septic response [4]. Animal studies in septic hamsters and pigs revealed that the administration of PCT was toxic and that neutralization by anti-PCT IgG antibodies significantly improved survival of affected animals [3, 27].

Functions of PCT in sepsis are not well clarified. One can suggests that PCT in sepsis could play some role in defence mechanisms intensifying the septic host response or to suppress the exaggerated immune response [28-30].

PCT has been shown to inhibit prostaglandin and tromboxan synthesis in lymphocytes *in vitro* and to

attenuate the LPS related stimulation of TNF-α production in whole blood cultures [30]. The pathogenesis of the septic shock is mainly due to unregulated TNF-α production at the beginning of sepsis. It was found that calcitonin gene--related peptid (CGRP) an alternative transcription products of the calcitonin gene inhibit TNF-α synthesis in rats [29]. Monneret et al. examined the role of PCT and CGRP in the regulation of the inflammatory response during the septic shock using a human whole blood model. They found that both PCT and CGRP had significantly inhibited the LPS induced TNF-α production. An antagonist of the CGRP receptor reversed the effect of CGRP. No effect on IL-1, IL-6 and IL-8 was found. This was the first description of an anti-inflammatory role for PCT and CGRP in humans [29].

Our results show that rhPCT increased the activity of unstimulated autologous lymphocytes, but decreased the activity of the PHA-preactivated T cells (by 14-26%) as well as the simultaneous activation of T cells with both rhPCT and PHA (by 21%). RhPCT decreased also the ability of T cells to be stimulated by Con A either simultaneously (by 13-19%) or subsequently (by 21-41%), what indicates that procalcitonin may downregulate the activation of T-lymphocytes. The influence of rhPCT on the activity of B cells was not uniform. Our results show for immunomodulatory activity of rhPCT. They show also some dichotomy which concerns the influence of rhPCT on the activity of lymphocytes. Our results are in line with works concerning the inflammatory activity (influence of rhPCT on the function of unstimulated lymphocytes) but also antiinflammatory activity of PCT (influence of rhPCT on the activity of stimulated lymphocytes). We haven´t found any other works concerning the influence of PCT on the mitogenic activity of lymphocytes so we cannot compare them. From this point of view our work could be considered as first of this kind.

Results with similar dichotomy were obtained by Wiedermann et al. (2002) who studied the influence of PCT and calcitonin (CT) on the migration and chemotaxis of monocytes [30]. They found that both PCT and calcitonin (CT) had elicited a dose dependent migration of monocytes – they acted as chemoattractants. However, PCT inhibited the chemotaxis towards formyl-Met-Leu-Phe (fMLP) a known chemoattractant. Stimulatory activity of PCT on migration (spontanneous locomotion) of monocytes contrasted with its effect on chemotaxis (directed locomotion) of these cells. Based on these results, Monneret et al. (2003) examined the activity of N-PCT and CGRP on LPS-induced expression of CD11b, one of the major integrins involved in monocyte and neutrophil diapedesis during a response to microbial infections. They found that both peptides had decreased the LPS- and fMLP-induced rise in CD11b in monocytes and neutrophils in a dose dependent manner [31]. These findings demonstrated the anti-inflammatory properties for this family of peptides. CGRP and calcitonin precursors may function as factors suppressing the propagation of inflammation through the inhibition of several processes involved during a response to a bacterial stimulus.

Little bit contradictory are also the results of Hoffmann et al. who investigated the possible association of PCT on inducible nitric oxid synthase (iNOS) gene expression and the release of nitric oxide (NO) in an *in vitro* cell culture model. Finally they conclude that rhPCT inhibits the iNOS-inducing effects of TNF-α/IFN-γ [31] and suggest that, although ineffective as an initiator of inflammation, PCT acts as a modulator of the inflammatory cascade, thus intensifying the septic host response [28].

Our results show a stimulatory activity of rhPCT for unstimulated autologous lymphocytes. The influence of rhPCT on the function of activated cells was different. RhPCT decreased the function of PHA-activated lymphocytes as well as the concurrent or subsequent activation of these cells in the presence of PHA. RhPCT decreased also ability of lymphocytes to be stimulated by Con A either simultaneously or subsequently. As both PHA and Con A predominantly activate T cells we can assume that rhPCT exerts a strong inhibitory activity preventing their hyperactivation.

We can suppose that the role of procalcitonin is probably not uniform. We suppose at least dual role for PCT on lymphocytes – PCT acts probably differently on unstimulated and activated T cells. The aim of procalcitonin released at the very early phases of systemic infection might thus be a potentiation of the immune response to the infection (rhPCT increased the mitogenic activity of unstimulated lymphocytes) in order to cope better with host invaders, whereas in a state of exaggerated immune system the role of PCT might be opposite (rhPCT decreased the mitogenic activity of stimulated T-lymphocytes) in order to protect the body from the toxic effect of hyperactivated immune system [34, 35]. We suggest that one of biological roles of PCT could be a downregulation of over-active inflammatory processes. However, we lay a question at the same time, if too intensive immunosuppressive effect of PCT could not be one of the reasons of immunodepression or immunoparalysis that are typical for the later phase of sepsis. So what´s a role of PCT in septic patients – helping or destroying, or both? Our results show for immunomodulatory activity of rhPCT but lot of experiments from many laboratories are still needed to obtain more precise and comprehensive results that finally clarify the complex view on the function of PCT in human body.

Conclusion

Our results show that rhPCT increases the activity of unstimulated autologous lymphocytes, but decreases the activity of PHA preactivated T cells (by 14-26%) as well as the simultaneous activation of T cells with both rhPCT and PHA (by 21%). RhPCT decreases also the ability of T cells to be stimulated by Con A either simultaneously (by 13-19%) or subsequently (by 21-41%), what indicates that procalcitonin may downregulate the activation of T-lymphocytes. We suggest that one of the biological roles of PCT could be a down-regulation of over-active inflammatory processes, including a suppression of hyperactive T cells.

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